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ITIM receptors: more than just inhibitors of platelet activation

Carmen H. Coxon[1], Mitchell J. Geer[2] and Yotis A. Senis[2]

[1]Target Discovery Institute, NDM Research Building, University of Oxford, Old Road Campus, Headington, OX3 7FZ

[2]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom B15 2TT

C.H.C. and M.J.G. contributed equally

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Key Points

Platelets express a variety of ITIM-containing receptors, each of which plays a unique role in regulating platelet function.

ITIM-containing receptors do more than inhibit ITAM-containing receptor signaling in platelets.

Abstract

Since their discovery, immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors have been shown to inhibit signaling from immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors in almost all hematopoietic cells, including platelets. However, a growing body of evidence has emerged demonstrating that this is an over-simplification, and that ITIM-containing receptors are versatile regulators of platelet signal transduction, with functions beyond inhibiting ITAM-mediated platelet activation. PECAM-1 was the first ITIM-containing receptor identified in platelets and appeared to conform to the established model of ITIM-mediated attenuation of ITAM-driven activation. PECAM-1 was therefore widely accepted as a major negative regulator of platelet activation and thrombosis for many years, but more recent findings suggest a more complex role for this receptor, including the facilitation of $\alpha_{IIb}\beta_3$-mediated platelet functions. Since the identification of PECAM-1, several other ITIM-containing platelet receptors have been discovered. These include G6b-B, a critical regulator of platelet reactivity and production, and the non-canonical ITIM-containing receptor TLT-1, which is localized to α-granules in resting platelets, binds fibrinogen, and acts as a positive regulator of platelet activation. Despite structural similarities and shared binding partners, including the Src homology 2 domain-containing protein-tyrosine phosphatases Shp1 and Shp2, knockout and transgenic mouse models have revealed distinct phenotypes and non-redundant functions for each ITIM-containing receptor in the context of platelet homeostasis. These roles are likely influenced by receptor density, compartmentalization, and as yet unknown binding partners. In this review, we discuss the diverse repertoire of ITIM-containing receptors in platelets, highlighting intriguing new functions, controversies and future areas of investigation.

Introduction

Platelets are small anucleate fragments derived from megakaryocytes that are vital for maintaining hemostasis. A growing body of evidence has established that platelets also contribute to other pathophysiological processes, including atherogenesis,[1] inflammation,[2] wound repair,[3] angiogenesis,[4] blood-lymphatic vessel separation[5] and cancer metastasis.[6] A common underlying theme is that platelets must transition from a *'resting'* to an *'activated'* state to contribute to each of these processes. Thus, understanding how platelets undergo this transition has broad implications for a number of diseases.

Taking thrombosis and hemostasis as a paradigm of platelet activation, transition from the *resting* state relies on key receptors coming into contact with their cognate ligands in the vessel wall and transmitting activation signals within platelets. Once adhered, thrombus formation is initiated and shaped accordingly to minimize blood loss, whilst maintaining blood flow. Tyrosine kinase-linked receptors (TKLRs), including the von Willebrand factor (vWF) receptor complex glycoprotein (GP)Ib-IX-V and the integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ play critical roles in the initial tethering, adhesion and activation of platelets at sites of injury. More robust activation is provided by the immunoreceptor tyrosine-based activation motif (ITAM)-containing collagen receptor complex GPVI-FcR γ-chain,[7] collagen-mediated clustering of which leads to phosphorylation of tyrosine residues within the FcR γ-chain ITAM by Src family kinases (SFKs). The Src homology 2 (SH2) domain-containing protein-tyrosine kinase (PTK) Syk can then be recruited and propagate the signal through tyrosine phosphorylation and a rise in intracellular Ca^{2+}. The cellular consequences of these series of biochemical events is the release of secondary mediators, including adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2), integrin activation and procoagulant activity. Secondary signals provided by the G protein-coupled receptors (GPCRs) for ADP ($P2Y_1$ and $P2Y_{12}$), TxA_2 (TP) and thrombin (PAR-1 and PAR-4), synergize with initiating signals from TKLRs

to maximally activate platelets and coordinate the platelet response with the coagulation system.

Equally important, but less well understood, are the mechanisms that prevent unwarranted platelet activation and limit thrombus size. Under normal physiological conditions platelets are maintained in a resting state by transient inhibitory signals that prevent unnecessary activation, whilst at the same time allowing platelets to fully respond to vascular injury when required. Two of the most powerful platelet inhibitors are prostacyclin (PGI_2) and nitric oxide (NO), which are released by healthy endothelium. They act by elevating intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels in the platelet via adenylate cyclase and soluble guanylate cyclase, respectively. These cyclic nucleotides in turn activate protein kinase A (PKA) and PKG, respectively, which phosphorylate key targets and inhibit platelet activation (Figure 1).[8]

Immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors provide a more specialized, cell-intrinsic inhibition, compared with PGI_2 and NO, by primarily targeting ITAM-containing receptors (Figure 2). The prototype of this family of receptors is the immune receptor FcγRIIB, which inhibits signaling from the ITAM-containing B cell receptor complex following ligand-mediated co-clustering of the two receptors.[9] Structurally, all ITIM-containing receptors belong to the immunoglobulin receptor superfamily, and carry at least one consensus ITIM sequence in their cytoplasmic tail, defined as I/V/LxYxxL/V (x represents any amino acid), in reference to the previously described ITAM (consensus sequence $YxxI/Lx_{6\text{-}12}YxxI/L$).[10] Whereas tyrosine phosphorylation of ITAMs provides docking sites for SH2 domain-containing Syk and the structurally-related PTK Zap70 in immune cells, tyrosine phosphorylation of ITIMs provides docking sites for SH2 domain-containing lipid- or protein-tyrosine phosphatases (PTPs), including SHIP-1, Shp1 and Shp2. The canonical mode of action of ITIM-containing receptors is positioning phosphatases in

close proximity to ITAM-containing receptors, such that the phosphatases are able to dephosphorylate and inactivate key components of the ITAM-containing receptor signaling pathway (Figure 2).

A third ITIM-like motif, termed an immunoreceptor tyrosine-based switch motif (ITSM, consensus sequence TxYxxV/I),[11] was subsequently described that confers activating and/or inhibitory properties to a receptor, depending on the associated signaling proteins. ITSMs were first identified in the signaling lymphocyte adhesion molecule (SLAM) CD150, which has both activating and inhibitory functions.[12] SLAM family proteins recruit members of the SLAM-adaptor protein (SAP) family, an interaction that is enhanced, but not dependent on, ITSM phosphorylation.[13] When SAP proteins associate with SLAM family receptors activating signals generally predominate, through recruitment of SFKs, whilst the absence of SAP adaptors allows phosphatase recruitment to promote inhibitory signaling.[13] The abundance and availability of the SAP adaptors adds a layer of complexity to ITSM-mediated control of SLAM receptor activity. ITSMs are often found in close proximity to ITIMs, facilitating interactions with SH2 domain-containing phosphatases, and contributing to the inhibitory function of ITIM-containing receptors.

For many years, PECAM-1 (also referred to as CD31) was the only known ITIM-containing receptor in platelets.[14-17] However, with the advent of proteomics and transcriptomics, several other structurally distinct ITIM-containing receptors were identified in platelets. These regulate various aspects of platelet function and include G6b-B,[18,19] TREM-like transcript (TLT-1),[20] carcinoembryonic antigen-related cell adhesion molecule 1 and 2 (CEACAM1 and CEACAM2)[21,22] and LILRB2 (also referred to as PIR-B),[23] structural features of which are summarized in Figure 3, and phenotypes of knockout (KO) and transgenic mouse models in Table 1. In addition, the inhibitory collagen-binding ITIM-containing receptor LAIR-1, also shown in Figure 3, has been identified in immature

megakaryocytes, but not mature megakaryocytes or platelets.[24] Recent work from our group has revealed that mice lacking LAIR-1 exhibit a mild thrombocytosis and increased proplatelet formation *in vitro*.[25] Interestingly, platelets produced by LAIR-1 KO mice exhibit an enhanced reactivity to collagen, despite not expressing the receptor, suggesting that effects are transmitted from megakaryocytes to platelets. Two ITSM-containing SLAM family members, CD150 and CD84, have been identified in platelets, as have the SAP family proteins SAP and EAT-2.[26-28] However, investigation of CD150 and CD84 KO mouse models revealed no overall effect on hemostasis, implying that their contribution to platelet function is minimal, and can be compensated for.[28,29]

With the recent increase in the number of ITIM-containing receptors reported in platelets and their diverse functional roles in regulating platelet homeostasis, we compiled this review to summarize structural and functional features, landmark discoveries, controversies and intriguing new findings that dispel the notion of ITIM-containing receptors as simply inhibitors of ITAM-mediated signaling (Figure 4). We focus on PECAM-1, G6b-B and TLT-1, which together illustrate the old, the new and the non-conventional members of this family of receptors in platelets.

PECAM-1 – the prototype

Structure and function

PECAM-1 is expressed on the majority of non-erythroid hematopoietic cells, including platelets, monocytes, neutrophils, T cells, and B-cell subsets, as well as being highly expressed on vascular endothelial cells.[30] This 130 kDa receptor has a large, highly glycosylated extracellular domain comprised of six immunoglobulin (Ig)-constant 2 (C2)-like domains, a transmembrane domain, and a cytoplasmic tail that conveys signaling functionality. Alternative splicing of *Pecam1* gives rise to a number of isoforms, with the

Δ15 isoform being the most prevalent in human platelets. PECAM-1 undergoes *trans*-homophilic interactions mediated by the two N-terminal IgC2-like domains, D1 and D2,[31,32] with particular dependence on Lys89[30], and an intracellular component encoded by exon 14 confers the capacity for heterophilic binding. A recent crystallographic study has revealed how D1 and D2 permit homophilic interactions in both *cis* and *trans*, and also how sugar moieties are accommodated at the binding interface.[33] Other factors, including receptor density, glycosylation and Ca^{2+}, can all influence PECAM-1-ligand interactions.[34] This interaction is likely to be of low affinity under resting conditions, as platelets do not adhere to each other or endothelial cells under healthy conditions, making platelet-platelet and platelet-endothelial cell interactions during thrombus formation the probable physiologically relevant context. Avidity and affinity are likely increased under these conditions. *In vivo* thrombosis models support this hypothesis as platelet PECAM-1 serves to limit thrombus size,[35] discussed in detail below. However, low affinity interactions under resting conditions cannot be discounted and may serve to strengthen inhibitory signals from PGI_2 and NO. Heterophilic binding partners include the integrin $\alpha_V\beta_3$,[36] neutrophil CD177[37] and CD38,[38] which are reportedly relevant in the context of inflammation and cancer.

Adjacent to the transmembrane domain of human PECAM-1 on the cytosolic side is a palmitoylation site (C595) followed by a proline-rich region (PRR), ITIM (Y663) and ITSM (Y686) – C594, Y662 and Y685, respectively, in murine PECAM-1 (Figure 3 and Table 2). Ligand engagement results in tyrosine phosphorylation of the ITIMs by SFKs, notably Lyn in platelets,[39] leading to recruitment of Shp1 and Shp2. The structure, phosphorylation and accessibility of these regions to binding partners are influenced by interactions with the plasma membrane.[40,41]

The primary function of platelet PECAM-1 is to inhibit platelet activation and thrombosis at sites of vascular injury by attenuating signaling from the ITAM-containing

receptor complex GPVI-FcR γ-chain when it engages collagen. This inhibition can however be overcome with high concentrations of GPVI agonists.[42] Although less general and less robust than endothelial-derived PGI_2 and NO,[43] the inhibitory effect of PECAM-1, together with that of other ITIM-containing receptors, is likely to be highly relevant in the context of atherosclerosis, when loss of the healthy endothelium results in a reduction in the local concentration of both of these soluble mediators. In addition to its recruitment of Shp1 and Shp2 to attenuate activating signals, there is evidence that PECAM-1 can sequester activating SH2 domain-containing proteins, including SFKs and the lipid kinase phosphatidylinositol 3-kinase (PI3K).[44] PECAM-1 also contributes to integrin $\alpha_{IIb}\beta_3$ outside-in signaling,[45] and to GPIbα internalization in a α_{IIb}-dependent manner.[46]

Our knowledge of how PECAM-1 modulates signaling and cell activity underpins much of what we know about ITIM-containing receptor signaling in platelets, and has expedited the characterization of new members of this family in this biological context.

Mouse Models

Studies using PECAM-1 KO (*Pecam1*$^{-/-}$) mice have greatly enhanced our understanding of the physiological functions of PECAM-1. Platelets isolated from *Pecam1*$^{-/-}$ mice display potentiated aggregation, Ca^{2+} release and secretion in response to collagen, the GPVI-specific ligand collagen-related peptide (CRP) and thrombin.[17,43] Paradoxically, platelets from *Pecam1*$^{-/-}$ mice spread less well on a fibrinogen-coated surface and exhibit reduced clot retraction compared with control platelets, suggesting that PECAM-1 is a positive regulator of $\alpha_{IIb}\beta_3$-mediated outside-in signaling.[45] However, the mechanism underlying this potentiation of integrin-mediated responses in platelets remains unclear, only a reduction in focal adhesion kinase phosphorylation has been shown. The observed phenotype could therefore simply be due to up-regulation of compensatory mechanisms that limit platelet

spreading on fibrinogen in the absence of PECAM-1. Further work is needed to determine the exact role of PECAM-1 in regulating $\alpha_{IIb}\beta_3$ signaling and function.

These changes in platelet reactivity result in enhanced thrombus formation following laser- and ferric chloride-induced injury in mesenteric arterioles and carotid arteries of mice, respectively, with both onset of thrombus formation and thrombus stability being increased.[35] Use of bone marrow chimeric mice proved this to be primarily due to loss of platelet, rather than endothelial PECAM-1.[35] In contrast, tail bleeding times were increased in *Pecam1*$^{-/-}$ mice, due to a loss of endothelial PECAM-1.[47] This suggests both positive and negative roles of PECAM-1 in thrombosis and hemostasis, respectively, dependent upon the lineage of cell expression. It should be noted that a separate study reported no difference in thrombus formation or bleeding between wild-type and *Pecam1*$^{-/-}$ mice following photochemical-induced injury of arterioles and venules and tail-bleeding assay.[48] However, there is little if any collagen exposure in this thrombosis model, instead it is predominantly dependent upon reactive oxygen species-mediated damage of endothelial cells.[49] In contrast, ferric chloride injury results in endothelial denudation and exposure of the basement membrane therefore increasing reliance upon GPVI-mediated platelet activation.[50] Lack of standardization and variability between different models likely accounts for the discrepancies between studies,[51] but also highlights the specialized role of PECAM-1 in these processes.[43] Regardless of these controversies, evidence clearly demonstrates a vital role for PECAM-1 in regulating platelet activation and function, involving both canonical and non-canonical ITIM-mediated signaling mechanisms.

With regards to platelet turnover, *Pecam1*$^{-/-}$ mice have normal platelet counts,[47,52] but exhibit a delayed recovery following antibody-mediated platelet depletion, due to impaired platelet production from megakaryocytes in the bone marrow.[52] This impairment is attributed to a lack of polarity of the leading edge of migrating megakaryocytes, mediated by the

polarized compartmentalization of stromal-derived factor-1α (SDF-1α) chemokine receptor CXCR4, and increased adhesion to extracellular matrix proteins *in vitro*.[53] As a consequence, PECAM-1-deficient megakaryocytes exhibit aberrant spatial distribution in the bone marrow following antibody-mediated platelet depletion.[52]

The biological functions of PECAM-1 extend beyond that of simply regulating platelet and megakaryocyte function. Numerous studies have highlighted the importance of PECAM-1 in a broad range of cell types, including endothelial cells, hematopoietic and immune cells,[54] all of which contribute to the overall phenotype of *Pecam1*$^{-/-}$ mice.

G6b-B – more than just a platelet inhibitor

Structure and function

The identification of G6b-B came almost a decade after that of PECAM-1,[55] and establishing its role in platelet function took a further 10 years. Restricted to platelets and megakaryocytes, G6b-B is one of a number of predicted variants (A-E) that arise from differential splicing (Figure 5). These are either transmembrane-containing (G6b-A and -B) or potentially secreted (G6b-C, -D and -E).[56] G6b-A and -B are both expressed in human platelets and have identical extracellular and transmembrane domains, but only the -B isoform contains the consensus ITIM and ITSM. Two other isoforms, G6b-F and -G, have been predicted, but are likely not expressed as they contain intronic sequences within an alternative C-terminus.

All *G6b* isoforms have a single variable-type Ig-like (IgV) domain and are N-glycosylated (one predicted site in humans and two in mice).[19,56,57] Human G6b-B has a classical ITIM consensus sequence at the membrane proximal tyrosine (Y194) and an ITSM (Y220) at the C-terminus (Figure 5 and Table 2). Two PRRs in the juxtamembrane region may provide docking sites for SH3 domain-containing proteins, but this remains to be

proven. An inhibitory role for G6b-B was supported by the observation that phosphorylated G6b-B isolated from pervanadate-treated COS-7 cells co-immunoprecipitated with both Shp1 and Shp2,[56] interactions that have since been verified in both human and mouse platelets.[18,19,57,58] Antibody-mediated cross-linking of G6b-B also promoted Shp1 recruitment and attenuation of CRP- and ADP-induced platelet aggregation and Ca^{2+} release.[18]

In vitro assays showed G6b-B to bind the physiological anti-coagulant heparin with a predicted affinity of 30 nM,[59] comparable to the heparin-anti-thrombin III interaction,[60] assuming a 15 kDa average molecular weight for heparin. This interaction is unexpected as G6b-B lacks a heparin-binding consensus sequence.[59,61,62] However, the ectodomain of G6b-B has a theoretical pI > 10, most likely giving it a positive charge at neutral pH, which may account for the interaction with highly negatively charged heparin. Heparin is released by mast cells in the sub-endothelial matrix, and interacts with a variety of proteins, including plasma proteins, receptors and cytokines to modulate biological and physiological processes. The best known function of heparin is as a cofactor of antithrombin III, conferring a 1,000-fold increase in its inhibitory function of thrombin.[63] The physiological significance of the interaction of heparin with G6b-B remains to be determined.

Mouse Models

With the exception of glycosylation, both mouse and human G6b-B are very similar in terms of expression, amino acid sequence (71% identity, 79% similarity), tyrosine phosphorylation, and binding of Shp1 and Shp2.[57] To determine the physiological function of G6b-B, constitutive and conditional KO mouse models ($G6b^{-/-}$ and $Pf4\text{-}Cre^{+};G6b^{fl/fl}$, respectively) were generated.[57,64] Both were healthy, fertile and viable, with no overt growth or developmental abnormalities. However, in line with *in vitro* observations,[65] an inhibitory

role for G6b-B in regulating signaling from GPVI-FcR γ-chain and the hemi-ITAM-containing podoplanin receptor CLEC-2 was found.[57]

One of the most striking and unique features of *G6b*$^{-/-}$ mice is a dramatic reduction in platelet counts. This could be explained by a combination of reduced platelet recovery following antibody-mediated platelet depletion, supported by a reduction in *G6b*$^{-/-}$ megakaryocyte proplatelet formation, and enhanced platelet clearance, due to platelets being pre-activated and having surface antibodies bound, targeting them for destruction.[57] Together, these defects contributed to a 77% drop in platelet count. It is unlikely these effects are due to loss of G6b-B expression in other cell types as G6b-B is highly lineage specific (Figure S1).[66] This is supported by the fact that *G6b* constitutive and conditional KO mouse models exhibit the same phenotype.[57,64] Platelet surface receptor levels were also altered to varying degrees in *G6b*$^{-/-}$ mice, particularly GPVI, which was reduced by 82% relative to platelets from control mice. This was most likely attributable to increased receptor shedding mediated by the metalloproteinase ADAM10, which was increased by 36% in platelets from *G6b*$^{-/-}$ mice.

Collectively, platelet defects exhibited by *G6b*$^{-/-}$ mice culminated in increased bleeding rather than a prothrombotic phenotype that would be expected when deleting an inhibitor of platelet activation. Reduced platelet counts alone cannot fully explain the bleeding diathesis, as it has previously been shown that 80-90% reduction in platelet count has only mild bleeding and thrombotic consequences in mice.[67] However, when combined with reduced reactivity to collagen and thrombin, as is the case in *G6b*$^{-/-}$ mice, then increased bleeding is the outcome. Collagen was still able to elicit a reduced aggregation and ATP secretion response, owing to increased avidity and signaling through simultaneous binding of GPVI and the integrin $\alpha_2\beta_1$, whilst signaling via CLEC-2 was enhanced, validating G6b-B as a negative regulator of ITAM-containing receptor signaling. This was further demonstrated

using *Gp6*$^{+/-}$*G6b*$^{+/-}$ mouse platelets, which hyper-responded to GPVI agonists, compared with platelets from *Gp6*$^{+/-}$ mice that expressed comparable levels of GPVI.[57] Interestingly, antibody-mediated depletion of CLEC-2 in addition to deletion of *Gp6* only partially rescued the *G6b*$^{-/-}$ phenotype,[57] suggesting that G6b-B has additional undefined functions in megakaryocytes and platelets, independent of its inhibitory effect on ITAM-containing receptor signaling. One such function is to positively regulate integrin-mediated spreading, identified by reduced ability of *G6b*$^{-/-}$ megakaryocytes to spread on fibrinogen, collagen and fibronectin, and thrombin-stimulated *G6b*$^{-/-}$ platelets to spread on fibrinogen.[57] These studies highlight the complexity of G6b-B signaling and demonstrate a clear role for this receptor in platelet production and clearance that cannot be compensated for by other ITIM-containing receptors. It should be noted that Shp1 and Shp2 conditional KO mice partially phenocopied *G6b* conditional KO mice,[64] further supporting the hypothesis that these SH2 domain-containing PTPs are pivotal effectors of G6b-B. In addition, the recently described *G6b-B diY/F* knockin mouse model, in which tyrosine (Y) residues within ITIM and ITSM were mutated to phenylalanine (F) residues, uncoupling G6b-B from Shp1 and Shp2, recapitulated the *G6b*$^{-/-}$ phenotype.[68]

The physiological role of G6b-B has also now been validated in humans by Melhem and co-workers who characterized a family that exhibited an autosomal recessive thrombocytopenia.[69] Whole exome sequencing identified a nonsense mutation at the codon for residue C108 of human *G6b* resulting in a stop codon (p.C108*) and protein instability in transiently transfected K562 cells.[69] Patients exhibited severe thrombocytopenia, splenomegaly, increased number of megakaryocytes and fibrosis in bone marrow biopsies, similar to that reported in *G6b* KO and *G6b-B diY/F* mice.[57,68] Interestingly, patients harboring this mutation were not reported to have a bleeding diathesis, whereas *G6b*$^{-/-}$ mice did show increased bleeding in a tail injury model, suggesting subtle differences underlie the

phenotypes in humans and mice. Although causes of these defects reported in both species can be varied, it is worth noting that mice expressing human G6b-B do not exhibit any of these defects,[70] supporting the hypothesis that human and mouse G6b-B perform the same physiological functions and are critical regulators of platelet homeostasis.

Signal transduction

Considerable evidence exists to support the hypothesis that G6b-B is an important regulator of ITAM-containing receptor and integrin signaling, as well as platelet turnover. Similar to other ITIM-containing receptors, the current understanding is that G6b-B mediates its actions largely through recruitment of Shp1 and Shp2 to the plasma membrane, but there may be additional contributions from interactions with other effectors.[58] In a manner akin to the sequestration of Shp2/p85 complexes by PECAM-1,[44] it is possible that G6b-B may compete for effectors of ITAM-containing receptor signaling under resting conditions to prevent spurious activation signals.[58] However, these interactions have yet to be validated in platelets and the high affinity of G6b-B for Shp1 and Shp2 (nM range),[58] strongly suggest these interactions are likely to dominate.

Once recruited, PTPs are predicted to be activated and dephosphorylate key substrates, leading to an attenuation of tyrosine kinase signaling and cell activation. However, the stoichiometry and downstream effects are probably more nuanced. Interestingly, G6b-B is highly phosphorylated in resting platelets compared with other platelet ITIM-containing receptors.[19] The significance of this is not yet known, but it suggests that G6b-B may be transmitting weak inhibitory signals even under resting conditions.

The two PRRs in the juxtamembrane region of the cytoplasmic tail of human G6b-B may serve to provide spatiotemporal control of signal strength, onset or duration of signaling,

as seen with GPVI. A pool of active SFKs can rapidly phosphorylate the GPVI-FcR γ-chain receptor following ligand engagement. These SFKs are maintained in a primed state through the actions of the receptor-like PTP CD148,[71,72] which dephosphorylates the inhibitory tyrosine residue in the C-terminal tail of SFKs. Active Lyn and Fyn are constitutively associated with the GPVI membrane-proximal PRR via their SH3 domain. This interaction is important for the kinetics of GPVI signaling,[73] with loss of the PRR delaying the onset, but not extent, of signaling by GPVI.[74] Intriguingly, Fyn, Src and Syk were shown to associate with phosphopeptides corresponding to the tandem ITIM/ITSM of G6b-B,[58] possibly aiding regulation of Shp1 and Shp2 interaction and downstream signaling. However, this has yet to be confirmed in platelets.

Thus, the biological function of G6b-B extends beyond that of inhibiting ITAM-containing receptor signaling in platelets (Figure 4). Its potential for regulating platelet reactivity under resting conditions and apparent role in platelet production, coupled with specific megakaryocyte-platelet expression (Figure S1), offers a novel therapeutic approach for managing thrombotic and hemorrhagic risk. It is likely that the elucidation of its endogenous ligand will greatly enhance our understanding of its function.

TLT-1 – the non-conventional

Structure/Function

Initially identified as the inhibitory analogue of the triggering receptor expressed on myeloid cells-1 (TREM-1) family of immune receptors, TLT-1 is a type I single IgV-containing surface receptor with an intracellular ITIM and ITSM-like sequence (Figure 3 and Table 2).[75] Whilst TREM-1 recruits the ITAM-containing adaptor protein DAP12 via a positive lysine residue in its transmembrane domain,[76] TLT-1 lacks this residue. TLT-1 was hypothesized to negatively regulate TREM-1 activity, yet its expression in blood cells is restricted to

megakaryocytes and platelets,[20] which have no detectable TREM-1 by proteomics-based approaches, raising the possibility of alternative functions. TLT-1 is in fact the most abundant ITIM-containing receptor in human and mouse platelets, which are estimated to contain 14,200 and 154,769 copies per platelet by quantitative proteomics, respectively (Table 3).[26,27] However, unlike other platelet ITIM-containing receptors, TLT-1 is localized specifically in α-granules in resting platelets, and is up-regulated on the surface following thrombin-mediated activation.[20] Surprisingly, despite containing an ITIM and a non-consensus ITSM (Table 2 and Figure 3), TLT-1 was found to have activating functions in a transiently transfected RBL-2H3 mast cell line, which showed enhanced FcεRI-induced Ca^{2+} mobilization,[77] while in platelets, antibody-mediated blocking of TLT-1 inhibits aggregation.[78]

Platelet activation with thrombin results in the shedding of the ectodomain of TLT-1 referred to as soluble TLT-1 (sTLT-1), which is a competitive inhibitor of TREM-1 and has anti-inflammatory properties.[79] Indeed, sTLT-1 levels were substantially elevated in plasma from septic patients compared with healthy individuals, correlating with disseminated intravascular coagulation associated with sepsis.[80] Recombinant protein comprised solely of the ectodomain of TLT-1 was found to augment platelet aggregation and also to bind fibrinogen, suggesting fibrinogen is the physiological ligand of membrane bound TLT-1.[80]

Signal transduction

Phosphorylation of Y230 in the ITSM-like domain and Y266 in the ITIM of human TLT-1 is required for downstream signaling, the latter being critical for Shp2 recruitment in transiently-transfected pervanadate-treated RBL-2H3 cells.[77] An interaction with Shp1 was demonstrated in transiently transfected HEK293 cells upon discovery of the receptor,[75] but this was not verified by others.[77,80] Shp1 and Shp2 interactions have not been validated in

platelets, but pull-down assays using the cytoplasmic tail of the receptor did show an interaction with the ezrin/radixin/moesin (ERM) proteins in HEK293 and COS7 cells. The moesin interaction was also identified in human platelets by immunoprecipitation of full length TLT-1.[80] The ERM family links membrane proteins to the actin cytoskeleton and have important roles in lymphocyte activation and cytoskeleton remodeling during migration.[81] Interestingly, TLT-1 contributes to early actin polymerization,[82] indicating the ERM interaction may help to promote platelet activation. Thus, signaling via TLT-1 is distinct to that of other platelet ITIM-containing receptors.

Mouse Models

The KO mouse model of TLT-1 (*Treml1*$^{-/-}$) revealed a critical role for this receptor in dampening the inflammatory response and facilitating platelet aggregation at sites of vascular injury.[80] This work confirmed an activating role for TLT-1 in platelets, notably aggregation responses to thrombin, collagen, ADP and U46619 were reduced, as was binding to fibrinogen, with a concomitant increase in tail bleeding times.[80] This is partially mediated by fibrinogen binding and TLT-1 linking to the platelet cytoskeleton, and presumably also by facilitating activation signals through an undefined mechanism. TLT-1-deficient mice also exhibit a 20% reduction in platelet count, suggesting a role in platelet production and/or clearance.[80] Lipopolysaccharide (LPS)-treated *Treml1*$^{-/-}$ mice developed higher plasma levels of TNF and D-dimer compared with control mice and were more likely to succumb to LPS challenge, correlating with elevated levels of sTLT-1 in septic patients.[80] Despite the major advances in our understanding of the pathophysiological functions of TLT-1, several key questions remain, including the functional role of TLT-1 in platelets and megakaryocytes, how it signals and whether platelet TLT-1 regulates leukocyte localization and function.

Other ITIM-containing receptor contributors

In addition to PECAM-1, G6b-B and TLT-1, several other ITIM-containing receptors have been shown to regulate platelet activation and thrombus formation (Table 1). CEACAM1 and CEACAM2 are expressed on a variety of tissues, including platelets (Figure S1),[66] and get upregulated on the surface of activated platelets, suggesting intracellular pools.[21,22] Findings from KO mouse models showed that both CEACAM1 and CEACAM2 attenuate ITAM receptor-mediated responses, presumably via Shp1 and Shp2, but paradoxically positively regulate integrin-mediated responses,[21,22,83,84] a recurring theme for platelet ITIM-containing receptors. Larger and more stable thrombi following ferric chloride injury *in vivo* was also demonstrated in CEACAM1- and CEACAM2-deficient mice, indicating that the net functions of CEACAM1 and CEACAM2 is to limit thrombus growth. Platelet counts were normal in both CEACAM1- and CEACAM2-deficient mice, suggesting they are not required for platelet production and/or turnover under normal conditions. It would be interesting to determine whether deletion of both receptors has additive or synergistic effects on platelet activation and thrombosis phenotypes observed in single KO mice. However, the close proximity of the *Ceacam1* and *Ceacam2* genes on chromosome 7, makes generation of double-deficient mice technically challenging.[85] Controversially, CEACAM1 is not detected in human platelets by proteomics-based approaches and at low copy number in mouse platelets (868 copies per platelet). CEACAM2, found only in the murine genome, was also not detected in mouse platelets (Table 3).[26,27,86] The reason for this discrepancy is not known, but may simply be a reflection of low abundance, masking of peptides during mass spectrometry or misassignment of peptides to other proteins following detection.[87]

LILRB2 (also referred to as PIR-B) is the most recent addition to the platelet ITIM-containing receptor family, and transgenic mice lacking the intracellular tail are uniquely thrombocythemic.[23] Platelet aggregation and signaling was mildly enhanced in response to

CRP, again supporting a role in negatively regulating ITAM receptor signaling. It is noteworthy that PIR-B is not detected in either human or mouse platelets by proteomics-based approaches,[26,27] possibly due to low abundance, but raising questions about the platelet phenotype of PIR-B KO mice. Observed phenotypes may arise from indirect effects of ablating PIR-B in other lineages and impacting platelet production. This in fact applies to most of the constitutive ITIM receptor KO and transgenic mouse models studied to date as all are expressed in other lineages, with the exception of G6b-B and TLT-1, which are highly megakaryocyte/platelet-specific (Figure S1).[66] Alternatively, because transcripts for all of these receptors are detected in the megakaryocyte lineage, observed platelet phenotypes may be due to defects in platelet production, or transfer of activating signals to platelets, as is the case in LAIR1 KO mice,[25] regardless of expression of the ITIM-containing receptor in platelets.

Summary

Platelets and megakaryocytes express an array of ITIM-containing receptors that vary in their abundance, structure and functional significance. KO and transgenic mouse models have been pivotal in establishing physiological functions of these receptors in the megakaryocyte lineage, but much remains to be explored. Despite all platelet ITIM-containing receptors interacting with Shp1 and Shp2, phenotypic analysis of mouse models are dramatically different and indicate additional roles beyond exclusively inhibiting ITAM receptor signaling (Figure 4). It is likely that there exists a spatiotemporal regulation system that governs receptor and effector availability and interaction, and that this system shapes distinct functional outputs. Shp1 and Shp2 are clearly major players in mediating the biological functions of ITIM-containing receptors in platelets, but the stoichiometry of binding and target specificity of these PTPs downstream of individual ITIM-containing receptors remains

to be determined. In addition, an activatory role in $\alpha_{IIb}\beta_3$ signaling, a temporally distinct event from signaling by GPVI-FcR γ-chain and CLEC-2, has been described for PECAM-1, G6b-B and CEACAM1/2. The mechanism by which these receptors facilitate integrin signaling remains to be determined. How TLT-1 acts as a positive regulator of platelet activation also has yet to be elucidated. A considerable amount of work remains to fully comprehend how ITIM-containing receptors work in concert with ITAM-containing receptors and integrins to orchestrate platelet reactivity and appropriate responses following injury, and how this may be overruled in pathological circumstances.

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Authorship

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Correspondence: Yotis A Senis, Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom B15 2TT; email: y.senis@bham.ac.uk

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Table 1. Summary of mouse platelet ITIM-containing receptors

Receptor	Characteristics	Mouse models	Signaling	References
PECAM-1 (CD31)	***Expression:*** non-erythroid hematopoietic cells, endothelium ***Isoforms:*** various, Δ15 most prevalent in human platelets ***MW:*** 130kDa ***Ectodomain:*** six IgC2-like domains, glycosylated ***Ligands:*** *trans*-homophilic, CD177, CD38, αvβ3 ***Intracellular motifs:*** palmitoylation (C594), PRR, ITIM (Y662), ITSM (Y685) ***Interactions:*** Shp1, Shp2, SHIP1, p85 (PI3K), Lyn	***Constitutive knock-out*** ***Platelet count:*** normal, delayed recovery following depletion ***Platelet volume:*** normal ***Receptor levels:*** normal ***Functional responses:*** ↑ collagen, thrombin aggregation ***Integrin function:*** ↓ spreading on fibrinogen, ↓ clot retraction ***Hemostasis:*** ↑ tail bleeding ***Thrombosis:*** ↑ onset, size, stability in laser and ferric chloride injury models	Attenuates ITAM, thrombin and integrin receptor signaling ↑ phosphorylation, Shp1 and Shp2 interaction to collagen, thrombin, fibrinogen and anti-PECAM-1 antibody stimulation	17, 30, 36, 37, 38, 39, 42, 44, 45, 47
G6b-B	***Expression:*** megakaryocyte and platelet specific ***Isoforms:*** membrane associated (-A and -B) and predicted secreted (-C, -D and -E) ***MW:*** 24-30kDa doublet, glycosylated ***Ectodomain:*** single IgV-like domain ***Ligands:*** heparin ***Intracellular motifs:*** PRR, ITIM (Y195), ITSM (Y221) ***Interactions:*** Shp1, Shp2, (Syk, PLCγ2 – only by SPR), Fyn>Src	***Constitutive and Pf4-Cre knock-out*** ***Platelet count:*** ↓ 77%, increased clearance and reduced production ***Platelet volume:*** ↑ 38% ***Receptor levels:*** ↓ 80% GPVI, minor ↓ α2, GPIbα and CLEC-2, ↑ ADAM-10 ***Functional responses:*** ↓ GPVI aggregation, ↑ CLEC-2 aggregation, thrombin aggregation reversible ***Integrin function:*** ↓ platelet spreading on fibrinogen with thrombin pre-activation, ↓ megakaryocyte spreading on fibrinogen, fibronectin and collagen ***Hemostasis:*** ↑↑ tail bleeding ***Thrombosis:*** unknown	Attenuates GPVI- and ADP-induced aggregation and Ca^{2+} release ↑ basal Syk phosphorylation (pY519/20) in *G6b*$^{-/-}$ platelets Basally phosphorylated ↑ phosphorylation, Shp1 and Shp2 interaction to CRP and thrombin	56, 57, 58, 59, 64
TLT-1	***Expression:*** megakaryocyte and platelet specific ***Isoforms:*** sTLT-1 (shed extracellular domain) ***MW:*** 38kDa ***Ectodomain:*** single IgV-like domain ***Ligands:*** fibrinogen ***Intracellular motifs:*** palmitoylation (C188), PRR, ITIM (Y266) ***Interactions:*** Shp1, Shp2, ERM proteins	***Constitutive knock-out*** ***Platelet count:*** ↓ 20% ***Platelet volume:*** unknown ***Receptor levels:*** unknown ***Functional responses:*** ↓ ADP and U46619 aggregation, delayed collagen and thrombin aggregation ***Integrin function:*** ↓ fibrinogen binding with ADP stimulation ***Thrombosis:*** unknown ***Hemostasis:*** ↑ tail bleeding ***Other:*** ↑ systemic inflammatory response to LPS	Augments platelet aggregation to multiple agonists Associates with ERM proteins potentially linking fibrinogen to the cytoskeleton	20, 75, 77, 78, 80

CEACAM1 (Bgp-1)	***Expression:*** immune, hematologic, epithelial and endothelial cells ***Isoforms:*** most dominant is CEACAM1L-4L (4 denotes the number of IgG domains, L denotes the long intracellular domain that contains the ITIMs) ***MW:*** 160kDa, heavily glycosylated ***Ectodomain:*** one IgV-like domain and three IgC2-like domains ***Ligands:*** homophilic ***Intracellular motifs:*** ITIMs (Y454) ITSM (Y481), PRR ***Interactions:*** Shp1, Shp2	***Constitutive knock-out*** ***Platelet count:*** normal ***Platelet volume:*** unknown ***Receptor levels:*** normal ***Functional responses:*** ↑ GPVI aggregation, granule release and flow adhesion ***Integrin function:*** ↓ platelet spreading on fibrinogen, ↓ clot retraction ***Hemostasis:*** ↑ tail bleeding ***Thrombosis:*** form larger, more stable thrombi	Attenuates GPVI and CLEC-2 mediated platelet activation – potentially via PLCγ2 Direct association and implicated in regulating function of αIIbβ3	21, 83
CEACAM2 (Bgp-2)	***Expression:*** platelets, intestinal tissue crypt epithelia, kidney, testis and several brain nuclei ***Isoforms:*** most dominant in platelets is CEACAM2-2L ***MW:*** 52kDa, glycosylated ***Ectodomain:*** one IgV-like domain and one IgC2-like domain ***Ligands:*** coronavirus mouse hepatitis virus spike glycoproteins ***Intracellular motifs:*** ITIM (Y273), PRR, ITSM (Y300) ***Interactions:*** Shp1, Shp2	***Constitutive knock-out*** ***Platelet count:*** normal ***Platelet volume:*** unknown ***Receptor levels:*** normal ***Functional responses:*** ↑ GPVI aggregation, granule release and flow adhesion, ↑ CLEC-2 aggregation ***Integrin function:*** ↓ platelet spreading on fibrinogen, ↓ clot retraction ***Hemostasis:*** ↑ tail bleeding ***Thrombosis***: form larger, more stable thrombi	Attenuates GPVI and CLEC-2 mediated platelet activation Augmented phosphorylation of PLCγ2, Src and Syk in response to CRP and rhodocytin in *ceacam2*$^{-/-}$ platelets	22, 84
PIR-B (LILRB2)	***Expression:*** platelets, immune cells (monocytes, macrophages and dendritic cells), hematopoietic cells and neurons ***Isoforms:*** Seven members ***MW:*** 125kDa, glycosylated ***Ectodomain:*** six IgC2-like domains, glycosylated ***Ligands:*** angiopoietin-like protein 2 (ANGPLT2) ***Intracellular motifs:*** ITIM (Y770), PRR ***Interactions:*** Shp1, Shp2	***Deletion of intracellular tail*** ***Platelet count:*** ↑ 47% ***Platelet volume:*** unknown ***Receptor levels:*** unknown ***Functional responses:*** ↑ GPVI aggregation and granule release, ↑ ADP and thrombin aggregation ***Integrin function:*** ↑ platelet spreading on fibrinogen ***Hemostasis:*** unknown ***Thrombosis:*** unknown	Attenuates collagen, thrombin and ADP induced platelet activation Attenuates Syk and FcRγ signaling upon antibody mediated cross-linking in immune cells ANGPLT2 attenuates GPVI-induced phosphorylation of LAT, SLP76, and PLCγ2 partially via PIR-B	23, 88

MW, molecular weight (Western blotting); Ig C2, immunoglobulin constant-2; PRR, proline-rich region; PI3K, phosphoinositide 3-kinase; Ig V, immunoglobulin variable; *Pf4-Cre*, platelet factor 4 promotor driven Cre-recombinase transgenic mouse. Residues are numbered according to the mature mouse protein after cleavage of the signal peptide.

Table 2. Megakaryocyte/platelet ITIM and ITSM receptor sequences

Receptor	Human			Mouse		
	ITIM	**ITSM**	**Non-consensus ITIM/ITSM**	**ITIM**	**ITSM**	**Non-consensus ITIM/ITSM**
PECAM-1	VQ**Y**$_{663}$TEV	TV**Y**$_{686}$SEV		VE**Y**$_{662}$TEV	TV**Y**$_{685}$SEI	
G6b-B	LL**Y**$_{194}$ADL	TI**Y**$_{220}$AVV		LH**Y**$_{195}$ADL	TV**Y**$_{221}$AVV	
TLT-1	VT**Y**$_{266}$ATV		TT**Y**$_{230}$TSL	VT**Y**$_{266}$ATV		SI**Y**$_{232}$TGS
CEACAM1	VT**Y**$_{459}$STL II**Y**$_{486}$SEV			VA**Y**$_{454}$TVL	TV**Y**$_{481}$SEV	
CEACAM2				VA**Y**$_{273}$TVL	TV**Y**$_{300}$SEV	
LILRB2 (PIR-B)	VT**Y**$_{541}$AQL		NL**Y**$_{512}$AAV SI**Y**$_{571}$ATL	VT**Y**$_{770}$AQL		SL**Y**$_{689}$ASV TE**Y**$_{746}$EQA SV**Y**$_{800}$ATL
LAIR-1	VT**Y**$_{230}$AQL IT**Y**$_{260}$AAV			VT**Y**$_{207}$IQL	ST**Y**$_{236}$AAI	
CD84		TI**Y**$_{258}$TYI TV**Y**$_{295}$SEV			TV**Y**$_{244}$AVV TI**Y**$_{279}$SSV	
CD150		TI**Y**$_{261}$AQV TV**Y**$_{307}$ASV	TI**Y**$_{287}$VAA		TI**Y**$_{264}$AQV TV**Y**$_{311}$ASV	TI**Y**$_{291}$VAA

ITIM, immunoreceptor tyrosine-based inhibition motif (consensus: I/V/LxYxxL/V); ITSM, immunoreceptor tyrosine-based switch motif (consensus: TxYxxV/I). Tyrosine residues are numbered according to the mature protein after cleavage of the signal peptide.

Table 3. Platelet ITIM- and ITSM-containing receptor protein and RNA expression levels

Receptor	Human		Mouse	
	Protein expression	**Gene expression**	**Protein expression**	**Gene expression**
PECAM-1	9400	42.45	5566	358.61
G6b-B	13700	638.61	29637	54.31
TLT-1	14200	568.68	154769	1118.59
CEACAM1	not detected	0.01	868	45.21
CEACAM2	N/A	N/A	not detected	0.41
LILRB2 (PIR-B)	not detected	0.63	not detected	0
LAIR-1	not detected	0.88	not detected	0
CD84	2900	7.60	9261	421.64
CD150	not detected	0.07	21750	312.32

Protein expression - estimated protein copy numbers per platelet in human[27] and mouse[26] quantitative proteomic studies; Gene expression – mean RPKMs (reads per kilobase of exon model per million mapped reads) values in human and mouse platelet quantitative transcriptomic studies[86]; N/A - gene not present in that species

Figure Legends

Figure 1. Inhibition of platelet activation by cyclic nucleotide generation. Prostacyclin (PGI_2) and nitric oxide (NO) activation of cyclic nucleotide generation providing generalized inhibition of platelet activation.

Figure 2. Classical inhibitory function of ITIM-containing receptors. Inhibition of ITAM-containing receptor signaling through the recruitment of the phosphatases Shp1, Shp2 or SHIP1.

Figure 3. Platelet ITIM-containing receptors. Main structural features are shown, including extracellular IgC_2-like and IgV-like domains and the main intracellular signaling motifs, namely ITIMs (consensus sequence I/V/LxYxxL/V), ITSMs (TxYxxV/I) and PRRs (PxxP) along with non-consensus ITIM/ITSM-like tyrosine residues. All receptors have been described in platelets with the exception of LAIR-1, which is only found in megakaryocytes. Residues are numbered according to mature mouse peptide sequences, following cleavage of the signal peptide.

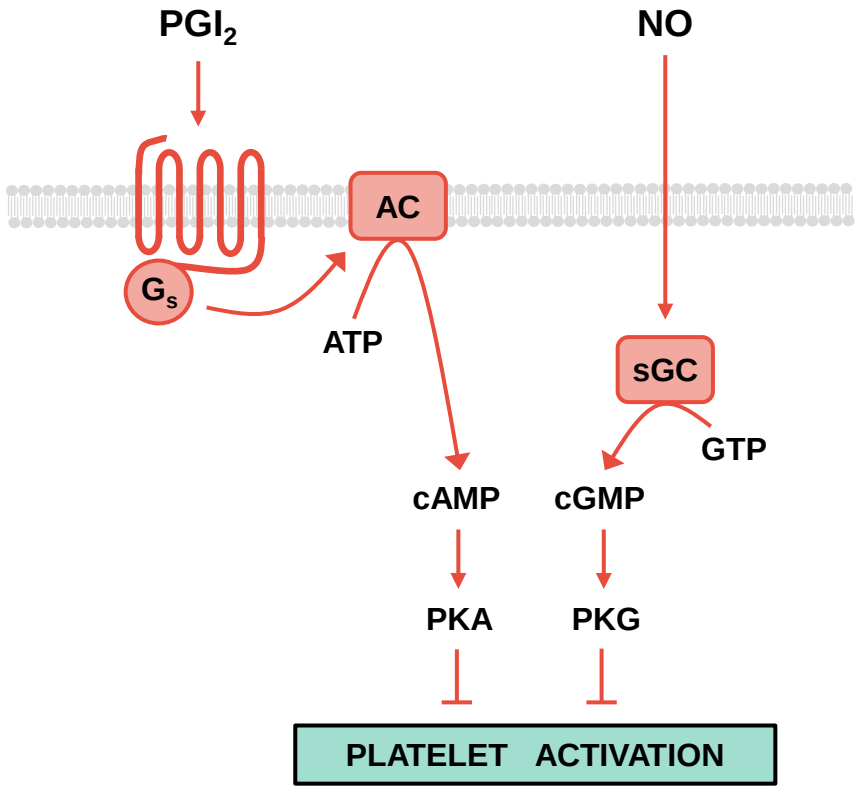
Figure 4. Classical and putative functions of ITIM-containing receptors. Increasing evidence is implicating ITIM-containing receptors as more than just inhibitors of ITAM-containing receptors, particularly as positive regulators of integrin-mediated functions. Potential roles in regulating cytoskeletal remodeling and G protein-coupled receptor (GPCR) signaling warrant further investigation.

Figure 5. Established and putative isoforms of human *G6b*. Main structural features are shown, including the IgV domain, ITIM, ITSM and PRR. G6b-A and -B contain transmembrane regions and are therefore represented as surface receptors. G6b-C, -D and -E, identified in transcriptome analysis but not as expressed protein, are predicted to be secreted

as they lack the transmembrane domain. Residues are numbered according to mature human peptide sequences, following cleavage of the signal peptide.

Figure S1. Gene-expression profile of platelet ITIM-containing receptors across major hematopoietic cell types in mice from Haemosphere database. Haemosphere (http://haemosphere.org/) online database was used to search Haemopedia dataset for (A) *Pecam1*, (B) *G6b*, (C) *Treml1*, (D) *Ceacam1*, (E) *Ceacam2*, (F) *Pirb* and (G) *Lair1* gene expression in primary murine hematopoietic cells.[66] Abbreviations: PreCFUE, PreColony forming unit - erythroid; MEP, Megakaryocyte Erythrocyte Progenitor; CFUE, Colony forming unit - erythroid; EryBlPB, Polychromatic erythroblasts (some Basophillic erythrobklasts); EryBlPO, Polychromatic erythroblasts (some Orthochromatic erythroblasts); Retic, Reticulocytes; Meg8N, 8N Megakaryocytes; Meg16N, 16N Megakaryocytes; Meg32N, 32N Megakaryocytes; Mast, Mast Cells; Baso, Basophils; EoP, Eosinophil Progenitor; Eo, Naive Eosinophil; NeutLN, Neutrophils from the lymph nodes; NeutPt, Neutrophils from the peritoneal cavity; MonoPB, Monocytes from the peripheral blood; MonoLN, Monocytes from the lymph nodes; Mac, Macrophage; CDP, Common DC Precursor; cDC1, Conventional Dendritic Cell 1; cDC2, Conventional Dendritic Cell 2; MigDC, CD205+ Migratory Dendritic Cell; ProB, B Cell Progenitor; PreB, B Cell Precursor; ImmB, Immature B Cell; B1, B1 B Cell; B2, B2 B Cell; MatB, Mature B Cell; CD4TThy1lo, Early Intrathymic CD4lo Precursor; TN1, Triple Negative Thymocyte 1; TN2, Triple Negative Thymocyte 2; TN3, Triple Negative Thymocyte 3; TN4, Triple Negative Thymocyte 4; DblPosT, Double positive thymocytes; NveCD4T, Naive CD4 T Cell; NveCD8T, Naive CD8 T Cell; EffCD4T, Effector Memory CD4 T Cells; EffCD8T, Effector CD8 T cells; CD4TLN, CD4 T Cell; CD8TLN, CD8 T Cell; RegT, Regulatory T Cell; MemCD8T, Memory CD8 T; NK, Resting NK cells.

Figure 1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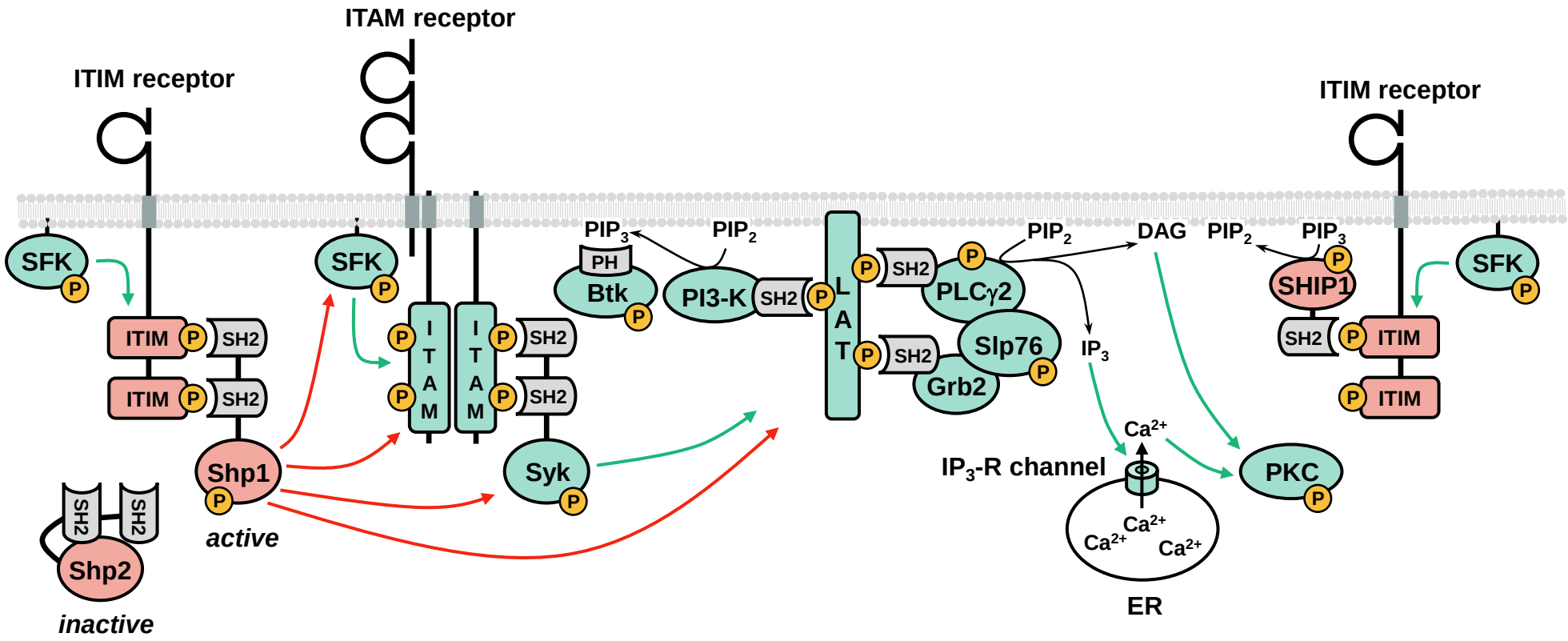
PKG
PLATELET ACTIVATION

Figure 2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ITIM receptor
ITIM receptor
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P
ITIM
SH2
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Btk
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IP3
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Figure 3

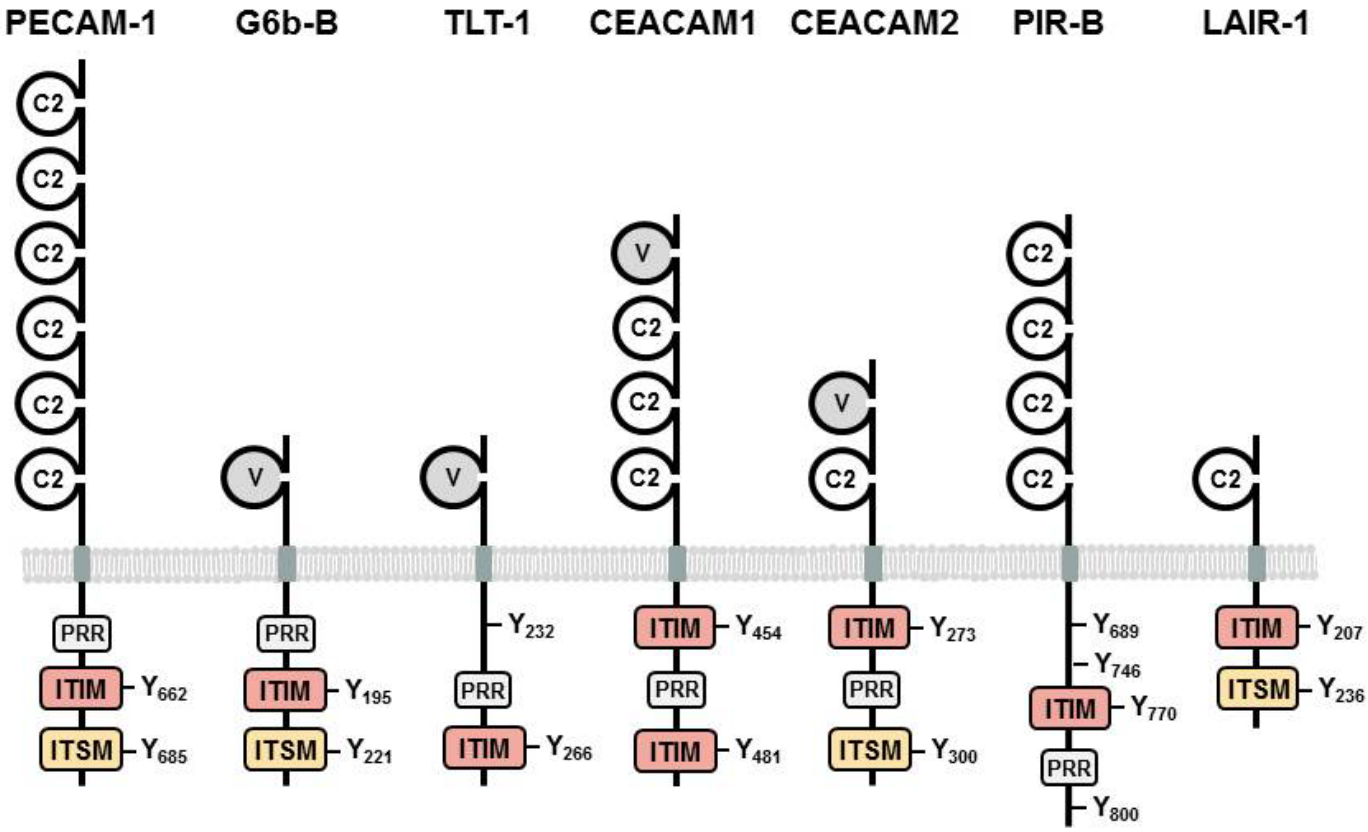
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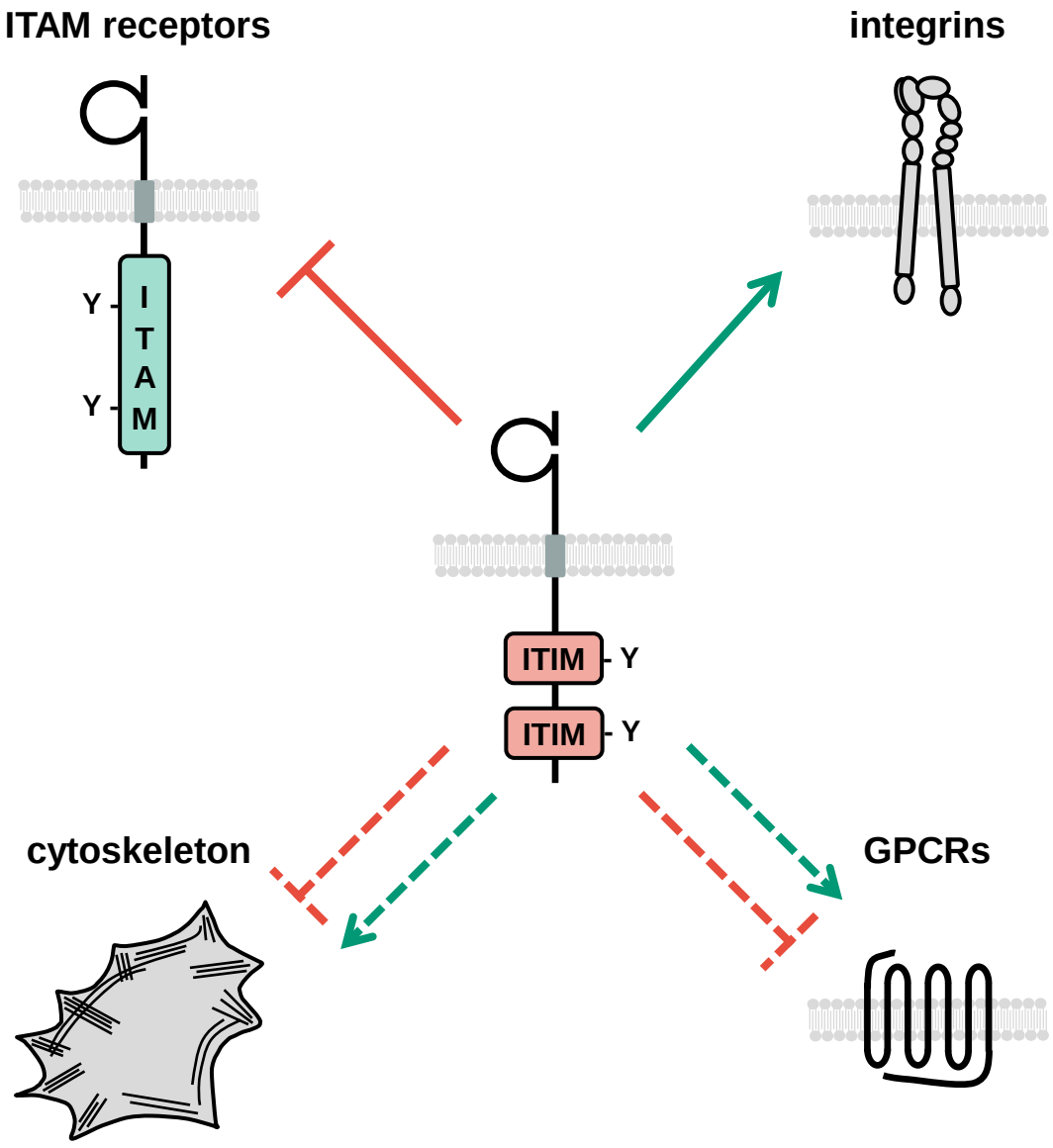
Figure 4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M
Y
ITIM
Y
ITIM
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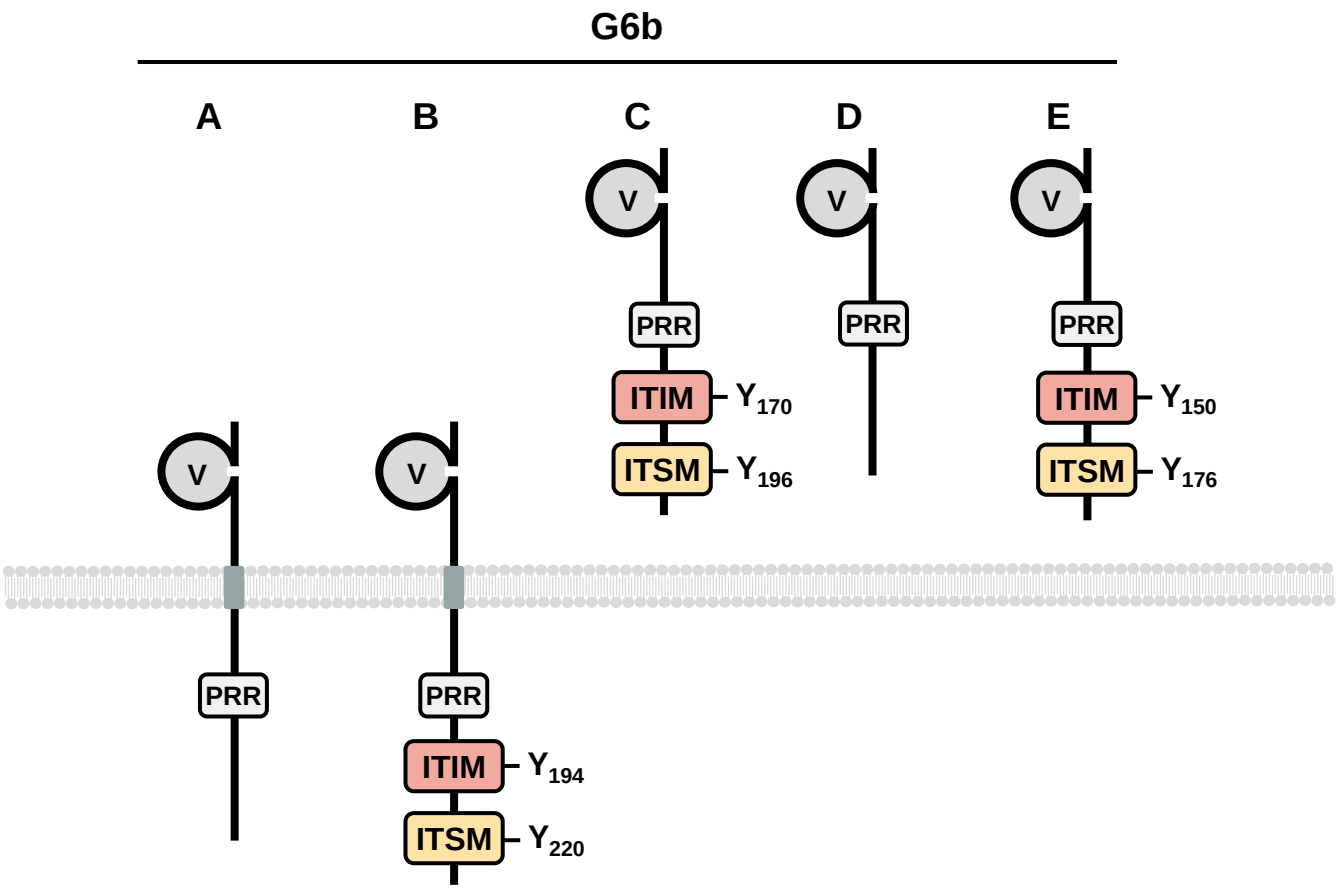
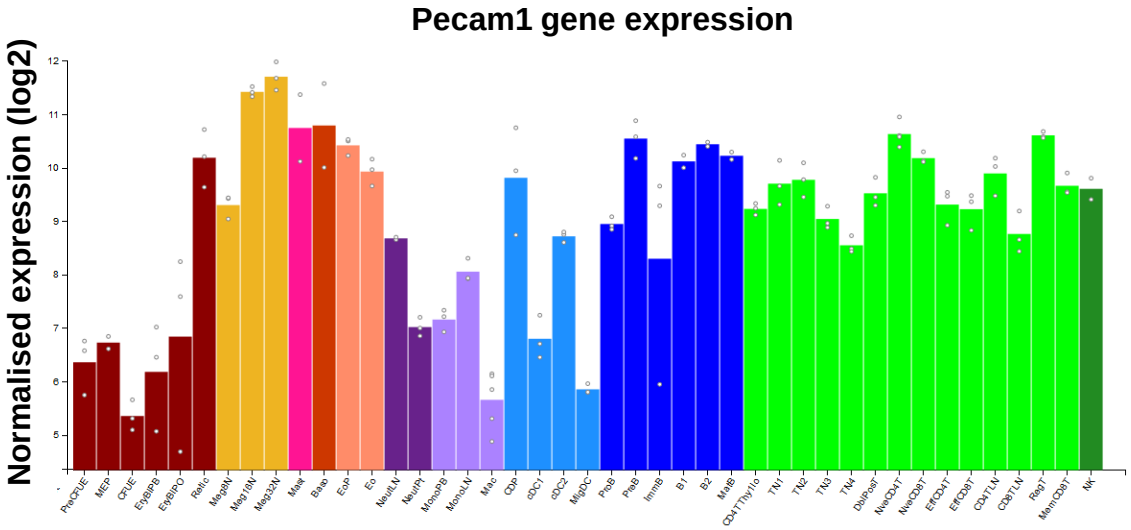
cytoskeleton
GPCRs

Figure 5



Supplemental Figure 1

A



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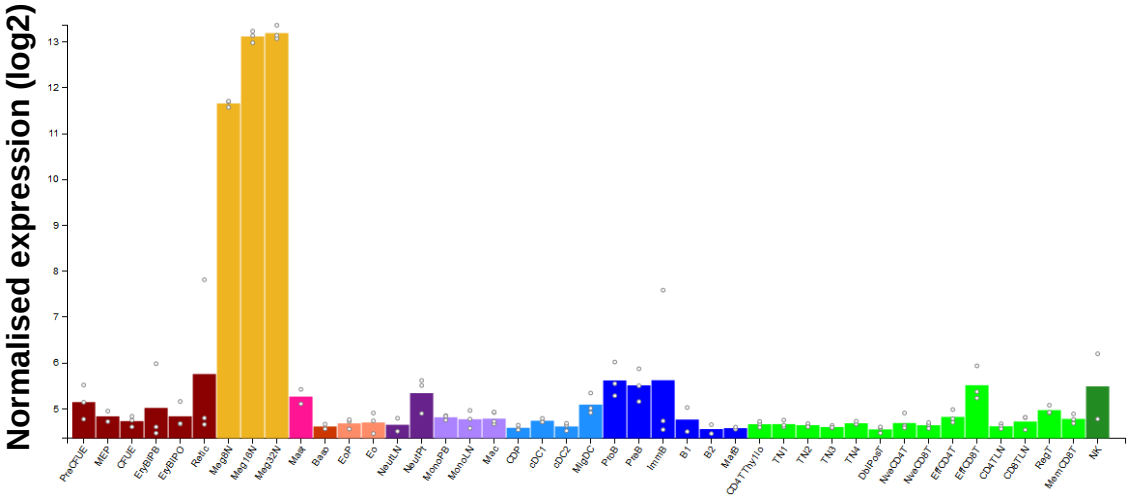

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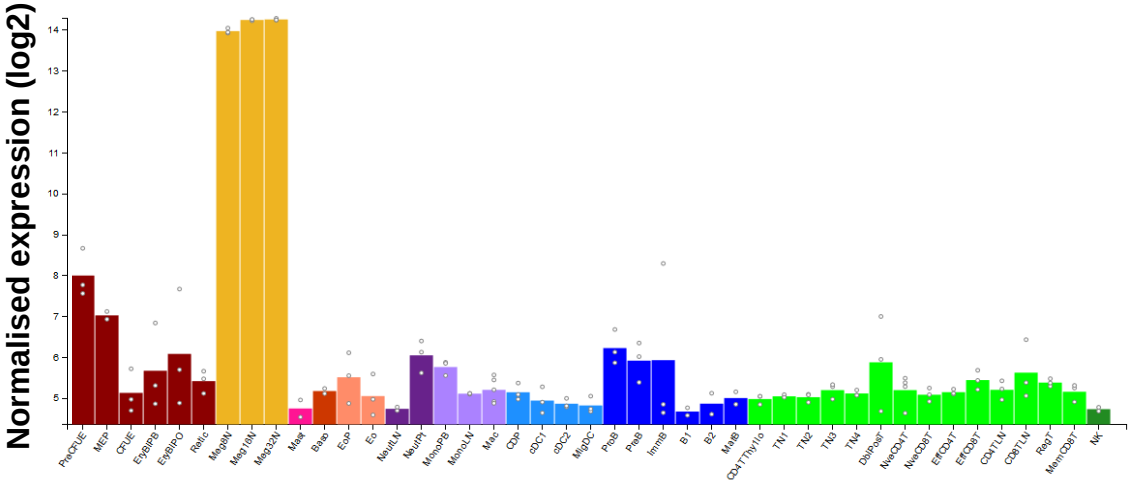
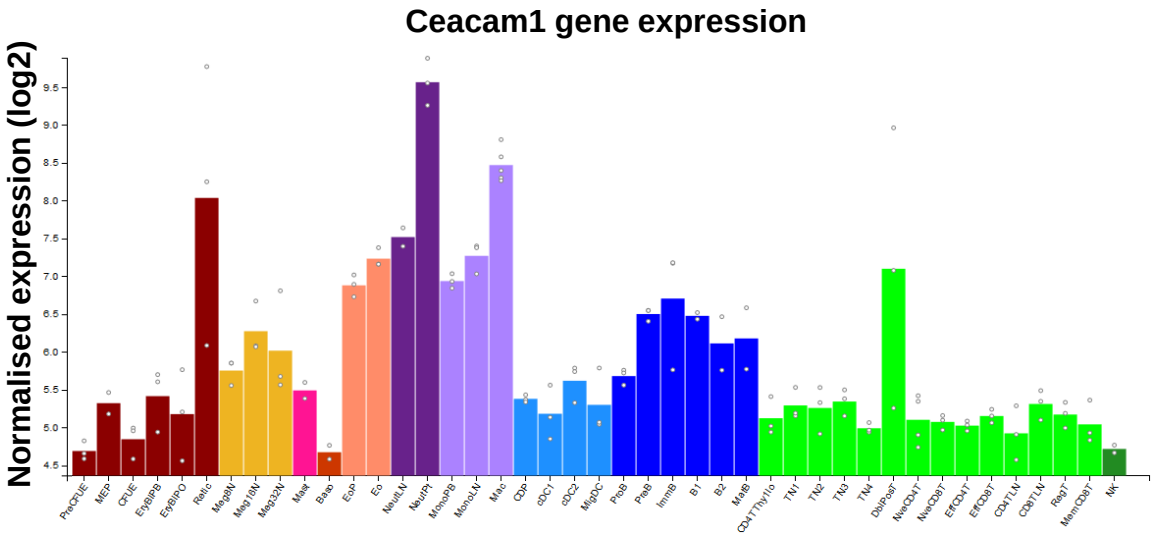


Supplemental Figure 1

D



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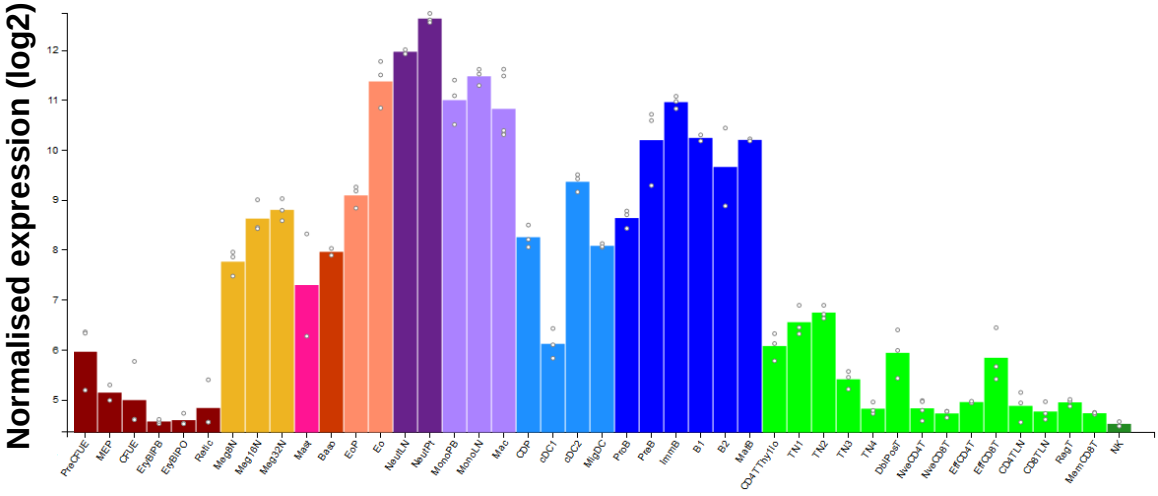

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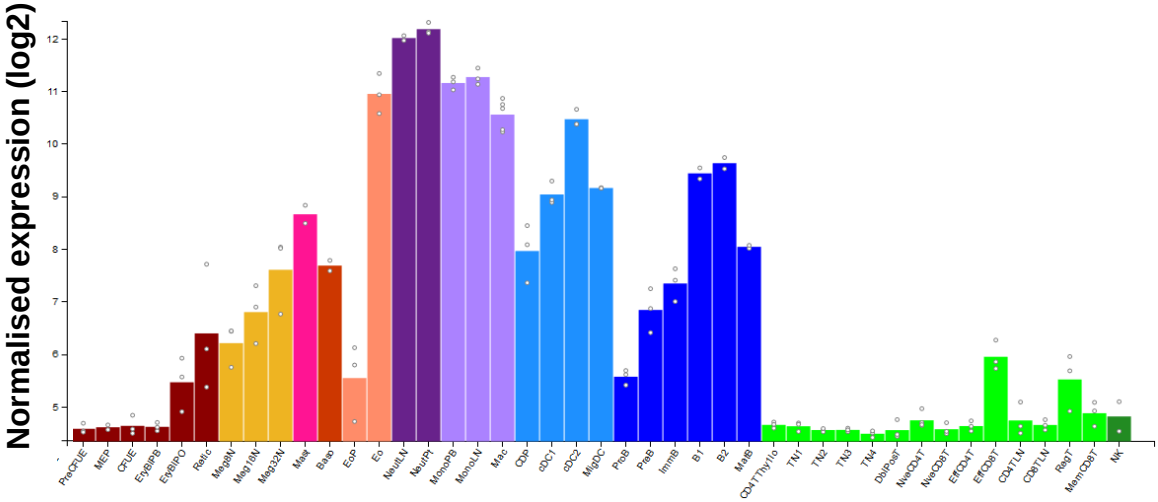

Supplemental Figure 1

G

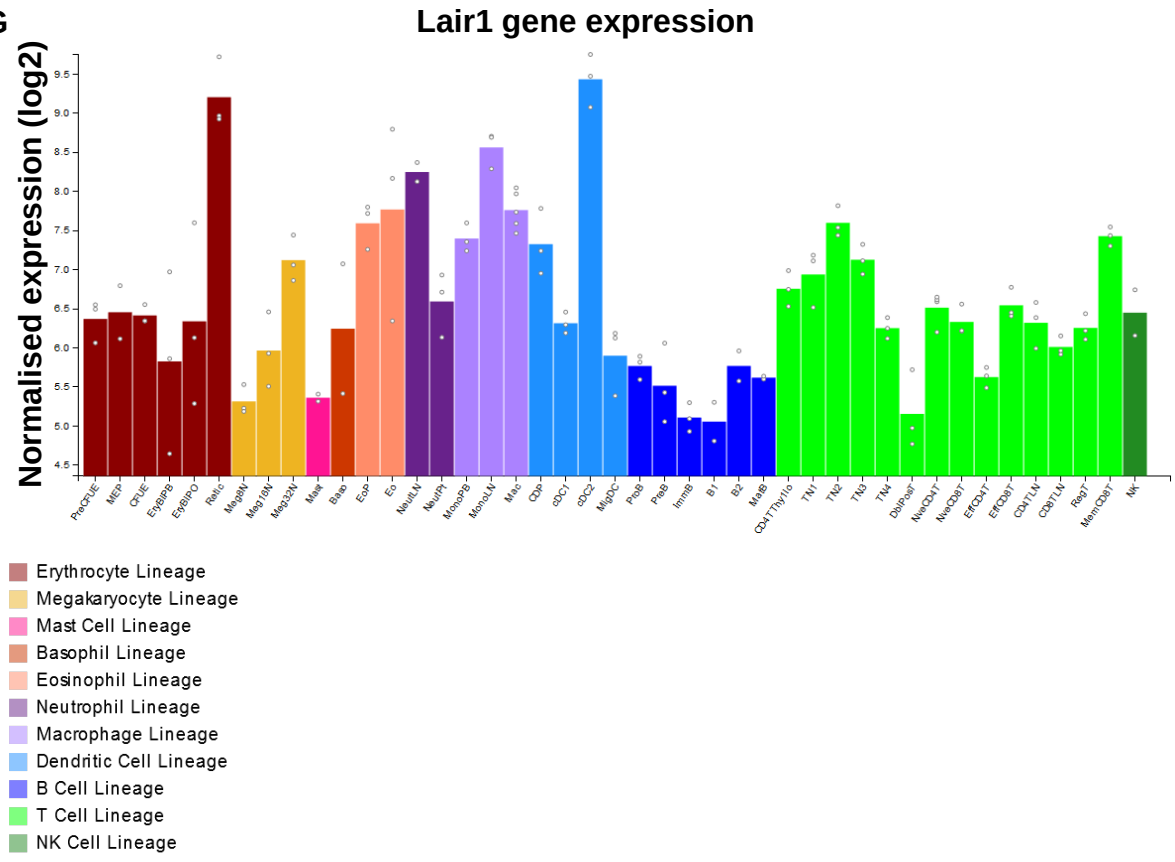

Figure S1. Gene-expression profile of platelet ITIM-containing receptors across major hematopoietic cell types in mice from Haemosphere database. Haemosphere (http://haemosphere.org/) online database was used to search Haemopedia dataset for (A) *Pecam1*, (B) *G6b*, (C) *Treml1*, (D) *Ceacam1*, (E) *Ceacam2*, (F) *Pirb* and (G) *Lair1* gene expression in primary murine hematopoietic cells.[66] Abbreviations: PreCFUE, PreColony forming unit - erythroid; MEP, Megakaryocyte Erythrocyte Progenitor; CFUE, Colony forming unit - erythroid; EryBlPB, Polychromatic erythroblasts (some Basophillic erythrobklasts); EryBlPO, Polychromatic erythroblasts (some Orthochromatic erythroblasts); Retic, Reticulocytes; Meg8N, 8N Megakaryocytes; Meg16N, 16N Megakaryocytes; Meg32N, 32N Megakaryocytes; Mast, Mast Cells; Baso, Basophils; EoP, Eosinophil Progenitor; Eo, Naive Eosinophil; NeutLN, Neutrophils from the lymph nodes; NeutPt, Neutrophils from the peritoneal cavity; MonoPB, Monocytes from the peripheral blood; MonoLN, Monocytes from the lymph nodes; Mac, Macrophage; CDP, Common DC Precursor; cDC1, Conventional Dendritic Cell 1; cDC2, Conventional Dendritic Cell 2; MigDC, CD205+ Migratory Dendritic Cell; ProB, B Cell Progenitor; PreB, B Cell Precursor; ImmB, Immature B Cell; B1, B1 B Cell; B2, B2 B Cell; MatB, Mature B Cell; CD4TThy1lo, Early Intrathymic CD4lo Precursor; TN1, Triple Negative Thymocyte 1; TN2, Triple Negative Thymocyte 2; TN3, Triple Negative Thymocyte 3; TN4, Triple Negative Thymocyte 4; DblPosT, Double positive thymocytes; NveCD4T, Naive CD4 T Cell; NveCD8T, Naive CD8 T Cell; EffCD4T, Effector Memory CD4 T Cells; EffCD8T, Effector CD8 T cells; CD4TLN, CD4 T Cell; CD8TLN, CD8 T Cell; RegT, Regulatory T Cell; MemCD8T, Memory CD8 T; NK, Resting NK cells.